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THE INHIBITION OF GLUTAMATE DEHYDROGENASE BY DERIVATIVES OF ISOPHTHALIC ACID

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Key Word Index—*Pisum sativum*; Leguminoseae; yeast; bovine; glutamate dehydrogenase inhibition; isophthalic acid derivatives.

Abstract—A range of compounds, structurally related to glutamate, have been tested as inhibitors of pea leaf glutamate dehydrogenase assayed in either direction. Only 5-*N*-substituted derivatives of aminoisophthalic acid completely inhibited the enzyme when tested at concentrations equal to either those of 2-oxoglutarate or glutamate. A minimum of three carbon atoms attached linearly to the amino group was required for maximum inhibition, inhibition was removed if there was any substitution on the first carbon. The 5-*N*-substituted derivatives also inhibited yeast (to a greater extent) and bovine liver (to a lesser extent) glutamate dehydrogenases.

INTRODUCTION

The precise physiological role of L-glutamate dehydrogenase (GDH) (EC 1.4.1.3) which catalyses the following reaction in higher plants has not been determined [1].

$$\text{2-Oxoglutarate} + NH_3 + \text{NAD(P)}H_2 \underset{(2)}{\overset{(1)}{\rightleftharpoons}} \text{L-Glutamate} + H_2O + \text{NAD(P)}.$$

There have been some suggestions that the enzyme may operate in the forward direction (1) under conditions of high ammonia levels [2, 3], or in the reverse direction (2) when proteins are being catabolized to yield carboxylic acids and asparagine [4].

A major contribution to determining the primary role of glutamine synthetase rather than glutamate dehydrogenase in ammonia assimilation has been the employment of methionine sulphoximine as a specific inhibitor of glutamine synthetase both *in vivo* and *in vitro* [1, 5]. The aim of this work was to identify an inhibitor of glutamate dehydrogenase that could be used in a similar manner.

The properties, regulation and role of glutamate dehydrogenases from micro-organisms and animals have been discussed expertly by Smith *et al.* [6]. A survey of inhibitors of the enzyme from these sources suggested that isophthalate and halogen derivatives of benzoic and furoic acids were potentially important compounds.

As far as we are aware, no details of any inhibitors of glutamate dehydrogenase from a plant source have been reported. As halogen derivatives would be expected to be generally toxic to plant cells and, thus, of little use for *in vivo* studies, the search for inhibitors was confined to derivatives of isophthalic acid.

RESULTS AND DISCUSSION

A range of compounds was tested as potential inhibitors of pea leaf GDH at a concentration equal to that of 2-oxoglutarate in the forward direction and to that of glutamate in the reverse direction (Table 1). Derivatives of 5-aminoisophthalic acid (as shown in Table 2) inhibited the enzyme 100% in both directions. No other class of

Table 1. The inhibition of pea leaf GDH by various glutamate analogues

Compound	% inhibition	
	Forward	Back
2,6-Pyridine dicarboxylate	82	57
m-Bromophenol	77	100
m-Bromobenzoate	59	93
5-Bromofuroate	58	88
2-Hydroxyaminoglutarate	48	86
5-Nitroisophthalate	29	100
Pentachlorophenol	25	100
2,4-Pyridine dicarboxylate	23	79
Glutarate	20	42
Isophthalate	18	76
3,5-Pyrazole dicarboxylate	16	76
4-Chloropyridine 2,6-dicarboxylate	13	22
3,5-Pyridine dicarboxylate	12	25
2-Fluoroglutarate	0	59
1,5-Dichloropentane	0	22
2-Amino-2-carboxycyclohexane acetate	0	20
1,1-Cyclohexane diacetate	0	18
Allylglycine	0	14
Pyridine tricarboxylate	0	10
1–9 (Table 2)	100	100

N-Acetylglutamate, 4-aminobutyrate, 4-amino-4-(5′-tetrazolyl)butanoate, 2-amino-5-hydroxyvalerate, *m*-hydroxybenzoate, 5-hydroxylysine, kainate, methionine sulphoximine, 2-methylglutarate 3-oxalyl-2,3-diamino propionate, *O*-phosphothreonine and propargyl glycine had no inhibitory action. Compounds were tested at 6 mM in the forward direction and 12 mM in the back reaction. GDH was assayed as described in the Experimental.

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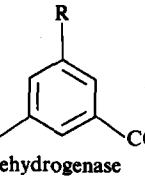
Table 2. Derivatives of isophthalic acid  employed as inhibitors of glutamate dehydrogenase

Compound	R	Name
1	$-NH-CH_2-CH=CH_2$	5-*N*-Allylamino-
2	$-NH-CH_2-C\equiv CH$	5-*N*-Propargylamino-
3	$-NH-CH_2-C(Me)=CH_2$	5-*N*-(2-Methylprop-3-ene)amino-
4	$-NH-CH_2-CH=CH-Me$	5-Crotonylamino-
5	$-NH-CH_2-CH_2-Me$	5-Propylamino-
6	$-N-(CH_2-CH=CH_2)_2$	5-*N*-Diallylamino-
7	$-NH-CH_2-C(Br)=CH_2$	5-*N*-(2-Bromoallyl)amino-
8	$-NH-CH_2-C(COOH)=CH_2$	5-*N*-(2-Carboxallyl)amino-
9	$-N_3$	5-Azido-
10	$-NH-CH(Me)-CH=CH_2$	5-*N*-(1-Methylallyl)amino-
11	$-NH-C(=O)-CH=CH_2$	5-*N*-Acryloylamino
12	$-NH-C(=O)-(CH_2)_3Cl$	5-*N*-(4-Chlorobutyryl)amino-
13	$-NH-C(=O)-Me$	5-*N*-Acetylamino-
14	$-NH-C(=O)-CH_2Br$	5-*N*-(Bromocetyl)amino-
15	$-NH-CH_2-CH_2OH$	5-*N*-(2-Hydroxyethyl)amino-
16	$-NH-C(=O)-CH_2-CH_2Cl$	5-*N*-(3-Chloropropionyl)amino-
17	$-NH-C(=O)-CH_2-Me$	5-*N*-Propionylamino-
18	$-O-CH_2-CH=CH_2$	-allyloxo-

compound inhibited the enzyme to the same extent. In fact, *m*-bromobenzoate, 5-bromofuroate, isophthalate and glutarate, which inhibit bovine GDH with K_is of 5.4×10^{-4}, 5.9×10^{-5}, 5.6×10^{-4} and 5.8×10^{-4} M, respectively [7], were only weak inhibitors of the plant enzyme.

5-*N*-Allylaminoisophthalate (**1**), the first compound of the series synthesized, was found to be a potent competitive inhibitor of pea leaf GDH (Fig. 1) with a K_i of 0.52 mM compared to a K_m for 2-oxoglutarate of 1.04 mM. A range of compounds was synthesized, based on 5-aminoisophthalate, and tested as inhibitors of the pea leaf enzyme and also of GDH isolated from yeast and bovine liver. Due to the small quantities of the compounds available, inhibition studies were confined to the forward direction only and the concentration required to obtain 50% inhibition with a 2-oxoglutarate concentration of 6 mM determined (Table 3).

It can be seen from Table 3 that a series of 5-*N*-substituted derivatives of aminoisophthalic acid are potent inhibitors of GDH from the three sources tested. The requirements would appear to be a minimum of a three carbon chain attached to the amino group. However, substituents on the first carbon atom (e.g. a keto or methyl group) tend to prevent the inhibitory action. Substituents on the second carbon atom (e.g. bromine on a carboxyl) or the presence of an unsaturated bond do not interfere with the binding of the compound to the enzyme. The presence of the nitrogen atom attached to the 5-position of the aromatic ring appears to be essential since when it was substituted by an oxygen atom in 5-allyloxoisophthalate (**18**) no inhibition could be detected.

Yeast GDH is more sensitive to the isophthalate derivatives than the bovine liver enzyme; K_i values determined for isophthalate for the *Neurospora* NAD

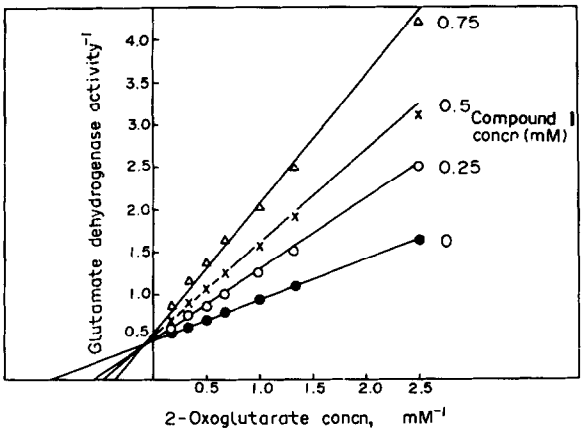


Fig. 1. Inhibitory action of 5-*N*-allylaminoisophthalate (**1**) on pea leaf GDH determined by the Lineweaver–Burk plot.

Table 3. Concentration (mM) of isophthalate derivatives required to inhibit by 50% the forward reaction of GDH isolated from three sources

Compound	Pea leaf	Yeast	Bovine liver
1	1.5	0.6	5.0
2	1.0	0.6	4.0
3	1.1	0.2	8.0
4	1.1	0.3	6.0
5	1.2	0.25	6.0
6	1.5	0.6	2.8
7	1.3	0.6	3.5
8	1.5	1.2	2.5
9	4.0	2.8	NI
10	NI	6.0	NI
11	NI	2.5	NI
12	NI	1.0	NI
13	NI	6.5	NI
14–18	NI	NI	NI

NI, No inhibition at 6 mM.

dependent enzyme (6.1×10^{-5} M) [8] and the bovine liver enzyme (5.6×10^{-4} M) [7] are consistent with this finding. The relative activity of all the compounds with the three different enzymes was, however, very similar. All the strong inhibitors fit into the model discussed by Fisher [9] that, in order to bind to the active site, the compounds must have two carboxyl groups 7.5 Å apart and preferably with an α-amino group.

As 5-*N*-allylaminoisophthalate (**1**) acts as a competitive inhibitor with a K_i value of only 0.52 mM, it is doubtful whether the derivatives would have any inhibitory actions if fed to plants at concentrations low enough to ensure that they were specifically acting on GDH only. However, it is clear that 5-aminoisophthalate may act as a lead compound for the synthesis of more potent inhibitors of plant GDH.

EXPERIMENTAL

Enzyme preparation. 20-day-old leaves of *Pisum sativum* var. Feltham First were extracted in 100 mM Tris–acetate (pH 8.2) containing 2 mM $CaCl_2$, 1 mM dithiothreitol and 10% (w/v) glycerol. The 50–65% $(NH_4)_2SO_4$ ppt was desalted on a Sephadex G25 column equilibrated in extraction buffer. Fresh yeast (*Saccharomyces cerevisiae*) was purchased from a local baker, frozen in liquid N_2 and extracted and partially purified as described above for the pea leaf enzyme. Bovine liver GDH (Type I) was purchased from Sigma.

Enzyme assay. The rate of the forward reaction was determined by measuring the disappearance of NADH at 340 nm in a spectrophotometer. The reaction mixture in 1 ml extraction buffer contained 6 μmol 2-oxoglutarate, 0.1 μmol NADH and 120 μmol $(NH_4)_2SO_4$. For the bovine liver enzyme, 0.2 M NaPi, pH 7.2, was employed in place of the Tris–acetate buffer.

The rate of the backward reaction was determined by measuring the appearance of NADH at 340 nm in a spectrophotometer. The reaction mixture in 1 ml extraction buffer contained 12 μmol glutamate and 1 μmol NAD.

Preparation of derivatives of isophthalic acid. 5-Azidoisophthalic acid (**9**) was prepared from 5-aminoisophthalic acid by the method of White and Yielding [10].

General method of preparation of 5-allylaminoisophthalates (**1–4, 6–8, 10, 11** *and* **15**). The substituted allyl bromide (0.1 mol) was added dropwise to a stirred soln of 5-aminoisophthalic (0.11 mol) in H_2O (100 ml) containing $NaHCO_3$ (0.33 mol). When only one layer remained (0.5–8 hr) the mixture was carefully made acidic using 40% HCl to give a white ppt which was collected.

General method of preparation of 5-amidoisophthalates (**1, 2, 13, 14** *and* **16**). Diethyl 5-aminoisophthalic acid was prepared from 5-aminoisophthalic acid by refluxing in EtOH containing conc. H_2SO_4. The appropriate acetyl chloride $(ClC(O)CH_2R)$ (0.1 mol) was added to the diester (0.11 mol) in $CHCl_3$ containing Et_3N (0.11 mol) with stirring and cooling. After 2 hr the $CHCl_3$ soln was washed with H_2O, dried over $MgSO_4$ and rotary evaporated to give a white solid which was recrystallized from Me_2CO–H_2O. Hydrolysis of the diester using KOH gave a pale yellow solid on acidification.

5-Allyloxyisophthalic acid (**18**). 5-Hydroxyisophthalic acid was synthesized from 5-sulphoisophthalic acid and the diester (0.1 mol) was then prepared as above and added to EtOH (100 ml) containing Na (0.11 mol). Allyl bromide (0.11 mol) was added and the mixture stirred and warmed for 3 hr. The EtOH was then removed and the residue partitioned between Et_2O and H_2O. The Et_2O layer was separated, dried over $MgSO_4$ and evaporated down to give a pale yellow solid. Hydrolysis of the solid with KOH gave a white solid on acidification.

5-Propylamino- and 5-propionylaminoisophthalic acid (**5** *and* **17**). Prepared by hydrogenation of **1** and **11**, respectively, using a Pd on Al_2O_3 catalyst at atm. pres.

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